

Comparison of three different methods for the presumptive detection of ESBL production in Ceftazidime-resistant strains of *Klebsiella pneumoniae*

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Abstract

Twenty-eight (28) strains of ceftazidime-resistant *Klebsiella pneumoniae* were isolated from blood cultures of in-patients from University Hospital, Kuala Lumpur between March 1995 and August 1996. Three methods were used to detect the production of ESBL enzymes by these strains. These three methods include the double-disc synergy test (DDST), inhibitor-potentiated disc-diffusion test (IPDD) and the E-test ESBL method. All strains could be identified as ESBL producers using the DDST method by a minimum of two beta-lactams and these included either a combination of ceftazidime and ceftriaxone with clavulanate respectively or cefotaxime and aztreonam with clavulanate respectively. Similarly using a combination of either cefotaxime and ceftriaxone with clavulanate or ceftriaxone and aztreonam with clavulanate respectively would have detected all strains as ESBL producers. The IPDD method could also detect for ESBL activity based on combinations of beta-lactam antibiotics with clavulanate respectively. All combinations of beta-lactam antibiotics could detect for ESBL activity in all the strains except a combination of either ceftazidime and aztreonam or cefotaxime and ceftriaxone with clavulanate respectively. The E-Test method using ceftazidime alone and in combination with clavulanate was found to be the most effective method in the presumptive identification of ESBL activity in all the strains.

Key words: *Klebsiella pneumoniae*, clavulanate, disc-diffusion, E-test, extended-spectrum β -lactamase.

INTRODUCTION

Members of the family *Enterobacteriaceae* producing extended-spectrum beta-lactamases (ESBLs) constitute a serious threat to current β -lactam therapy. These ESBLs are mainly mutants of their parental non-ESBL types which have evolved by point mutation resulting in each subtype having a single amino acid substitution thus resulting in increased resistance to the extended-spectrum beta-lactams. These parental beta-lactamases include TEM-1, TEM-2 and SHV-1 enzymes.^{1,2,3}

Strains of the family *Enterobacteriaceae* mainly produce ESBLs of the Molecular class A type which are inhibited by beta-lactam inhibitors such as clavulanate unlike the Molecular class C type which is not inhibited or poorly inhibited by clavulanate and is also produced in these strains.

The three methods to detect for ESBLs described in this paper is based on the inhibition by clavulanate and thus enabling the detection of ESBL enzymes of the class A type which is an applicable technique for the ESBL detection in *Enterobacteriaceae* family.

In vitro detection of ESBL expression has proved to be difficult because many of these strains are reportedly susceptible to the widely used and tested broad-spectrum β -lactams cefotaxime and ceftriaxone. Optimizing the detection of extended-spectrum β -lactamases (ESBLs) is important for microbiology laboratories because routine methods for monitoring a decrease in susceptibility to oxyimino-cephalosporins are not sensitive enough to detect all ESBL-producing strains, especially those that produce ESBLs like TEM-7, TEM-12 and SHV-2.⁴

A simple screening test for ESBL production is to test for synergy between ceftazidime and clavulanate. Ceftazidime is a good substrate for most ESBLs and is thus an appropriate indicator drug.⁵ A ≥ 16 -fold difference between the MIC of ceftazidime and that of the ceftazidime/clavulanate combination has been adopted to designate putative ESBL production among *klebsiellae*.⁶ However, MIC determination by dilution methods is labour intensive, so technically simpler alternatives are needed. The

objective of this study was to evaluate three methods the double-disc synergy test, inhibitor-potentiated test and E-test ESBL screen for the detection of ESBL production.

MATERIALS AND METHODS

Bacterial strains

Twenty-eight strains of ceftazidime-resistant *Klebsiella pneumoniae* (MIC > 128 mg/L) isolated from blood cultures of in-patients from University Hospital, Kuala Lumpur between March 1995 and August 1996 were studied. The isolates were identified as *Klebsiella pneumoniae* by standard biochemical tests. Ceftazidime which was used as a marker of resistance towards ESBL producing strains, was used in the screening test.

Detection of ESBL enzymes

Three methods were used to detect the production of ESBL enzymes by the ceftazidime-resistant *Klebsiella pneumoniae* strains and are described below. The tests are based on the inhibition of ESBL activity by β -lactamase inhibitors especially clavulanic acid. Strains were inoculated into peptone water and the turbidity was adjusted to 0.5 McFarland giving an equivalent bacterial concentration of 10^8 CFU/ml. Using a sterile cotton tipped swab the inoculum was lawned on a 90mm Mueller-Hinton agar (Amersham, USA) plate using the same method as was used for the disc diffusion technique as described by NCCLS.⁷

a. Double disc synergy test

The double disc synergy test (DDST) was carried out as described by Jarlier *et al.*⁸ Ceftazidime (30 μ g), cefotaxime (30 μ g), ceftriaxone (30 μ g) and aztreonam (30 μ g) discs were placed 25mm centre to centre away from a 20 μ g amoxicillin/10mg clavulanate disc before incubation. The plate was then incubated in an inverted position at 37°C overnight under aerobic conditions and observed for enhancement of the ceftazidime zone of inhibition near the amoxicillin-clavulanic acid disc producing a key-hole appearance. A positive result would indicate the production of ESBL enzymes by the test organisms.

b. E-test ESBL screen

E-test ESBL screen (TZ/TZL) strips were purchased from AB Biodisk, Sweden. The E-test screen is a plastic strip impregnated with ceftazidime alone (0.5mg/L-32mg/L) on one end and both ceftazidime (0.125mg/L-8mg/L)

and clavulanate, at a constant concentration of 4mg/L on the other.⁴

The plate was then incubated at 37°C overnight. The MIC value was read where the elliptical zone of growth inhibition intersected the E-test strip.

The MICs of ceftazidime alone on one end of the strip was compared with the MIC of ceftazidime/clavulanate combination at the other end. A greater than four fold reduction in the ceftazidime MIC in the presence of clavulanate was taken as positive for ESBL production.⁹

c. Inhibitor-potentiated disc-diffusion test (IPDD)

Four β -lactams i.e. ceftazidime, ceftriaxone, cefotaxime and aztreonam were tested for synergy with clavulanate. Mueller-Hinton agar supplemented with 4mg/L of clavulanate was prepared by the addition of clavulanate to molten MH agar after cooling to 50°C in a water bath. Antibiotic discs containing ceftazidime (30 μ g), cefotaxime (30 μ g), ceftriaxone (30 μ g) and aztreonam (30 μ g) were placed on 90mm clavulanate containing and clavulanate-free Mueller-Hinton agar plates. After overnight incubation at 37°C, the diameters of the inhibition zones around the antibiotic discs were measured.⁴

Augmentation zone widths were obtained by subtracting inhibition zone diameters produced by the β -lactam discs on clavulanate-free medium from those produced on clavulanate-containing medium. An augmentation zone width of \geq 10mm was considered positive for ESBL production.⁴

All the antibiotic discs used were purchased from Oxoid, USA except for aztreonam which was from BBL, Becton Dickinson, USA.

RESULTS

Double-disc synergy tests.

All strains were positive when tested for the presence of ESBL enzymes in the double-disc synergy test when specific combinations of the four antibiotics were used. Enhancement of the β -lactam disc zone diameter near the clavulanate disc indicated inhibition of the ESBL enzyme by clavulanate.⁸ β -lactam discs that were tested for synergy with clavulanate included ceftazidime, aztreonam, ceftriaxone and cefotaxime. However there were some strains that did not show presence of ESBL enzymes when only some of the β -lactam discs were used. These included 1 strain that did not show augmentation of both ceftazidime and cefotaxime with clavulanate and another strain that did not

show synergy of both ceftazidime and aztreonam with clavulanate. Two strains did not show augmentation of ceftazidime with clavulanate and another two did not show synergy with ceftriaxone and clavulanate. However, the use of a combination of at least two antibiotics, ceftazidime and ceftriaxone with clavulanate respectively or cefotaxime and aztreonam with clavulanate respectively would have detected all strains as ESBL producers. Similarly using the combination of cefotaxime and ceftriaxone with clavulanate or ceftriaxone and aztreonam with clavulanate would have detected all the strains as ESBL producers (Table 1).

E-Test ESBL Screen

The test identified all strains as ESBL producers. This was shown by the reduction in MIC of ceftazidime/clavulanic acid by greater than four-fold when compared to the MIC of ceftazidime alone. The log reduction of the strains ranged from 16 to 256 with 50% of the strains having a log reduction of > 64 (Table 2).

Inhibitor-potentiated disc-diffusion method

All strains were shown to be ESBL producers by the inhibitor-potentiated disc-diffusion test when a combination of antibiotics were used. Diameters of inhibition zones of β -lactam antibiotics on agar plates without clavulanate were measured and compared with inhibition zones of β -lactam antibiotics on agar plates incorporated with clavulanate. Augmentation zone widths of ≥ 10 mm was considered positive for ESBL production⁴. Augmentation zone widths of up to 24mm was observed in some strains. However three strains did not show

augmentation using some of the β -lactam antibiotics. These included one strain that did not show augmentation of zone diameters of ceftazidime and aztreonam in the presence of clavulanate respectively and another that did not show augmentation of zone of inhibition of cefotaxime and ceftriaxone. Yet another strain did not show the presence of ESBL when tested with ceftazidime and clavulanate only. In order to detect the presence of ESBL enzymes in all the strains a minimum of two β -lactam discs could be used relating to any of the combinations except a combination of either ceftazidime and aztreonam or cefotaxime and ceftriaxone (Table 3).

Comparison of the double-disc synergy, E-Test ESBL Screen and inhibitor-potentiated disc-diffusion methods in detecting ESBL enzymes

All the strains showed the presence of ESBL enzymes by the E-test ESBL screen. This indicates that this method is very reliable for the confirmation of ESBL producing strains. However the double-disc synergy method was not able to detect ESBL production in all the strains using only certain β -lactam antibiotics; using ceftazidime alone, four strains were not detected as ESBL producers by this test and 2 strains were not identified when ceftriaxone was used alone. Ceftazidime and aztreonam respectively with clavulanate would have also not detected 1 strain as an ESBL producer and one strain would not have been detected if only cefotaxime and ceftazidime were used respectively with clavulanate. As the inhibitor-potentiated disc-diffusion method is based on a quantitative method it is a more objective method

TABLE 1: Detecting for ESBL producers using 2 β -lactam combinations

Two β -lactam combination with clavulanate respectively	ESBL producers (n)	
	DDST	IPDD
CAZ + CRO	28	28
CTX + CRO	28	27
CRO + AZT	28	28
CTX + CAZ	27	28
CAZ + AZT	27	27
CTX + AZT	28	28

Key: CAZ= ceftazidime; CTX= cefotaxime; AZT= aztreonam; CRO= ceftriaxone

TABLE 2: Detecting for ESBL producers by E-Test MIC and their respective log reduction.

Strains (n)	E-Test MIC (mg/L) (caz/caz + clav)	Log reduction
1	16/0.5	32
1	32/0.25	256
14	>32/0.5	>64
9	>32/0.25	>128
1	>32/1	>32
1	16/0.25	64
1	>32/2	>16

Key: CAZ= ceftazidime; Clav = clavulanate

TABLE 3: Detecting for ESBL producers by comparison of the three different methods.

Antibiotic (with clavulanate respectively)	No. of strains detected as ESBL producers		
	DDST	IPDD	E-Test ESBL
CAZ	26	26	28
CRO	26	27	NA
CTX	27	27	NA
AZT	27	27	NA

Key: CAZ= ceftazidime; CTX= cefotaxime; AZT= aztreonam; CRO= ceftriaxone; NA = not applicable

compared to the double-disc synergy method which is a qualitative method. However using this method also does not ensure the confirmation of ESBL production in all the strains unless a combination of β -lactams are tested. ESBL production was not detected by this method in one strain using ceftazidime and aztreonam and in another strain using cefotaxime and ceftriaxone (Table 2).

DISCUSSION

In-vitro testing for the presence of ESBL enzymes was carried out using double-disc synergy test, inhibitor-potentiated disc-diffusion test and E-Test ESBL Screen. ESBL production was inferred by synergy between ceftazidime and clavulanate 4mg/L, based on the fact that all the extended-spectrum TEM and SHV derivatives give clear resistance to ceftazidime and are inhibited by clavulanate.

A test described by Jarlier *et.al.*⁸ based on synergy between β -lactamase inhibitors and

cefotaxime, ceftazidime, ceftriaxone or aztreonam enables the detection of ESBLs in strains misclassified as susceptible on the basis of standard interpretative criteria for the disc-diffusion method.

The double-disc synergy method although was a straight forward technique, could not detect ESBLs in all the strains. This method involves the placing of the augmentine disc and the ceftazidime disc at a distance of 25mm from centre to centre of the discs on an agar plate. The results obtained from this method were interpreted qualitatively and not quantitatively, unlike the other two methods mentioned. This could lead to misinterpretation of the results. Previous studies have also been reported using this method of ESBL detection but a distance of 30mm was maintained between the two discs as was reported by Abigail *et. al.*¹⁰ Therefore the distance between the discs was not a standard measurement and varied from 20mm to 30mm and was based upon the qualitative results obtained upon optimization. However there were

also strains that gave positive ESBL results when the disc distances were increased to 35 or 40mm as was reported by Ho *et. al.*⁴ Their study suggested that this method lacks sensitivity based on the test when ceftazidime alone was used that resulted in only 87% of the strains being ESBL positive. In order to avoid false negative results due to substrate specificity, it was suggested that a minimum of two β -lactam agents should be tested in this method, as was also evident from this study.

The E-test ESBL screen method however could detect ESBL production in all the strains. This method was also the simplest and fastest compared to the other two methods since it only involves the addition of the E-test strip onto the agar plate. The E-test strips are incorporated with ceftazidime antibiotic on one end and ceftazidime/clavulanate on the other end. By comparison of both their MICs the log reduction in the MIC could be tabulated quantitatively. Although this method was the most reliable method in detecting ESBL producing strains, not all laboratories may be able to afford to purchase the strips as these commercial strips may be expensive.

Among the three methods used to detect ESBL producing strains, the inhibitor-potentiated disc method was the most tedious method since agar plates incorporated with a specific concentration of clavulanate had to be prepared prior to the testing. Furthermore this method could not detect ESBLs in all the strains by only using a single β -lactam agent. In order to avoid false-negative results due to substrate specificity, the data presented indicated that a minimum of two agents should be used in this method. This was also supported by Ho *et. al.* where zone augmentations with ceftazidime for one strain expressing SHV-2 and another expressing SHV-3 were < 10mm. In contrast, the zone augmentations with cefotaxime for both strains were >10mm⁴. However this method is specific and highly sensitive since strains producing ESBLs could be clearly separated from non-producers by a breakpoint of \geq 10mm zone augmentation. Similar findings were also reported by Ho *et. al.*⁴. In their study, of the four β -lactams used, as was also used in the present study, ceftazidime was the most sensitive indicator drug for detecting ESBLs⁴.

Some ESBLs have 1 to 4 amino acid substitutions compared with their parent enzyme resulting in a remodeled active site to allow the attack on aminothiazolyl compounds thus resulting in different substrate specificities. These

enzymes mediate resistance to extended-spectrum cephalosporins and monobactams. However these enzymes vary considerably in the levels at which they express resistance to various cephalosporins such as ceftazidime and cefotaxime. SHV-derived enzymes appear to confer much higher resistance than do TEM-derived enzymes to most third and fourth generation cephalosporins.^{11,12}

Based on the three methods evaluated, E-test ESBL Screen was the most reliable method in detecting ESBLs. However the other two methods could also be used as long as a minimum of two agents were used to avoid false-negatives. Similar findings were reported by Ho *et. al.*¹ From the present study it would seem that either cefotaxime and ceftriaxone or aztreonam and ceftriaxone would be able to detect ESBLs in all strains using the double-disc synergy test. For inhibitor-potentiated disc-diffusion method any two β -lactam agents was acceptable except a combination of either ceftazidime and aztreonam or cefotaxime and ceftriaxone.

In the recent NCCLS guidelines⁷ it has been suggested that for the initial screening for ESBL producing strains, cefpodoxime disc be used as in addition to ceftazidime as this detects all ESBL producing strains and as a phenotypic confirmatory test both cefotaxime and ceftazidime, alone and in combination with clavulanic acid be used.

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